(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
03 October 2024 (03.10.2024)

WIPO | PCT

(10) International Publication Number
WO 2024/201521 A1

(51) International Patent Classification:
C12N 1/18 (2006.01) *C12R 1/865* (2006.01)

(21) International Application Number:
PCT/IN2024/050326

(22) International Filing Date:
27 March 2024 (27.03.2024)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
202341022068 27 March 2023 (27.03.2023) IN

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CV, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IQ, IR, IS, IT, JM, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, MG, MK, MN, MU, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, CV, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SC, SD, SL, ST,

(54) Title: TENDER COCONUT WATER-BASED MEDIUM FOR BAKER'S YEAST AND BIOETHANOL PRODUCTIONS

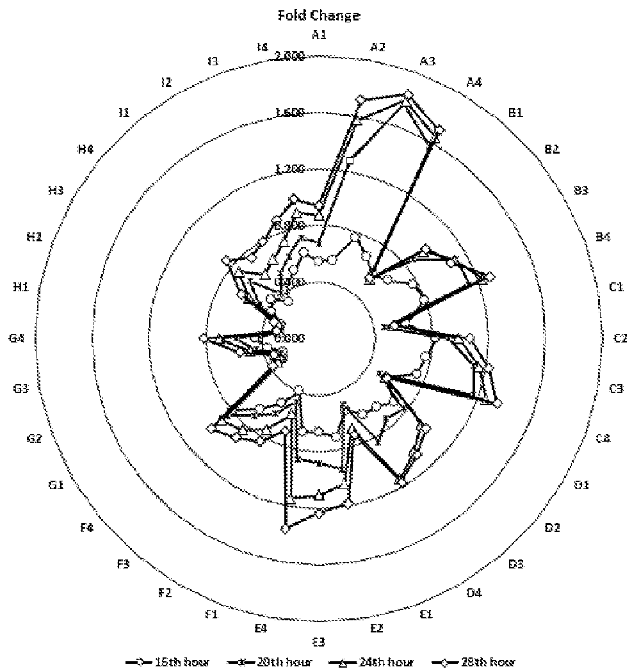

Fig. 19e

(57) Abstract: Baker's yeast, Saccharomyces cerevisiae is the major ingredient used for baking industries The quality and quantity of baker's yeast depend on the quality of the media used. Tender Coconut Media (TCW) is used as a nutritional media source for the production of baker's yeast. The present disclosure describes a method for producing optimized TCW media by supplementation with carbon and nitrogen source to produce good quality and quantity of baker's yeast for baking and ethanol production. Optimized TCW media supplemented with carbon sources such as glycerol, glucose, sucrose and fructose and nitrogen source such as ammonium sulphate yields a good quantity of cells at 135 hours for bread preparation and ethanol production. Maximum ethanol was obtained with a substantiated inoculum of 10% and 20% (v/v). The growth of yeast was found to be enhanced in the TCW-based medium as compared to YEPD media and Molasses.

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SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, ME, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:
— *of inventorship (Rule 4.17(iv))*

Published:
— *with international search report (Art. 21(3))*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*
— *in black and white; the international application as filed contained color or greyscale and is available for download from PATENTSCOPE*

TENDER COCONUT WATER-BASED MEDIUM FOR BAKER'S YEAST AND BIOETHANOL PRODUCTIONS

TECHNICAL FIELD

The present invention generally relates to tender coconut water (TCW)-based media for growing yeast such as baker's yeast (*Saccharomyces cerevisiae).* More specifically, the present invention relates to a method of producing optimized TCW media for production of high biomass of yeast (such as *Saccharomyces cerevisiae*) for baking and bioethanol production.

BACKGROUND

The lifestyle of humans is changing worldwide with the increasing consumption of fast food that includes ready to use (RTU), ready to eat (RTE) food, and bakery products. For the preparation of bakery products, the most essential raw material is baker's yeast (at least 2%). Baker's yeast is a commercial preparation consisting of dried cells of one or more strains of the fungus *Saccharomyces cerevisiae*. The baker's yeast has an extensive history of use in the area of food processing as a leavening agent in baking bread as a fermenter of alcoholic beverages and wine production. *Saccharomyces cerevisiae* plays a vital role during the fermentation step to get a preferred shape, texture and quality of the product. The quality of the raw material utilized should be good, and it depends on the growth media, optimum temperature, and the conversion process from wet to dry form.

The significance of yeast in food technology, as well as in human nutrition, as an alternative source of protein to overcome the demands in a world of low agricultural production and rapidly increasing population, makes the production of food-grade yeast quite important (Bekatorou et al. 2006).

In brewing industries, commonly, two species of yeast are used. *Saccharomyces uvarum, Saccharomyces cerevisiae. Saccharomyces cerevisiae* is the cheapest available strain used for bio-ethanol production (Bhadana and Chauhan 2016). *Saccharomyces cerevisiae*, in the presence of oxygen converts sugar into alcohol and carbon dioxide. Industrial bioethanol production plants employ mainly two types of primary feedstocks, viz., starch from cereal crops and juice or molasses from sugar crops (Mojović et al. 2006; Wilkie et al. 2000). About 60% of the global ethanol is produced from sugar crops, while the remaining 40% is produced from starchy grains (Salassi 2007).

Ethanol is primarily produced by the petrochemical process or by the yeast fermentation. *Saccharomyces cerevisiae* is capable of very rapid rates of ethanol production under optimal conditions (Dombek and Ingram 1986). The main disadvantage in using sugars as feedstock is low storability and microbial decomposition (Dodić et al. 2009), whereas downstream processing of starchy grains as a feedstock is too expensive (Azhar et al. 2017).

Tender coconut water (TCW) is a pure colorless liquid inside the coconut palm, which is nutrient-rich and a good source of vitamins, minerals, antioxidants, amino acids, enzymes and growth factors. Yeast is a microorganism that is commercially grown using synthetic growth media like YEPD, MPYA, SYPA, etc. Further, byproducts like molasses (cane, beet) or fruit wastes are commonly used as a carbon source for large scale yeast production.

Yeast used for the production of bakery products may have contamination of heavy metals due to the use of waste by products. For example, molasses contains some of the heavy metals like Al, As, Cu, Fe, Mn and Zn at a concentration of 0.54, 0.24, 8.7, 0.35, 11.1 and 19.7 μg/g, respectively (Teclu et al. 2009). An ideal alternative can be the utilization of a natural source that is free of chemicals and has natural nutrients required for the optimum growth of microorganisms. TCW has been used earlier as a media for the growth of bacteria (Oloke and Glick 2006; Sekar et al. 2013), mushroom, and also yeast (Sekar et al. 2013). It acts as a good carbon source for the organisms as it is rich in nutrients and also provides a good yield compared to the synthetic media. Yeast is used in starter cultures for the production of various types of fermented foods like bread, sourdoughs, fermented meat, vegetable products, etc. (Kandasamy et al. 2018).

Nowadays, the byproducts like molasses are becoming expensive due to the unavailability of enough sugarcane for its production, which is the result of drought and adverse climatic changes (Zhao and Li 2015); hence, natural sources are better and a cheap alternative as compared to byproducts. In addition, it has been used for biofuels production and other applications.

To overcome all these problems along with reducing the cost of chemicals to the manufacturer as well as reducing the side effects for the consumers, there exists a need for preparation of an optimized green media for the production of yeast of high biomass as well as for pharmaceutical grade bioethanol production.

SUMMARY OF THE INVENTION

Consequently, there is a need for an organic/green culture medium for production of yeast of high biomass as well as for production of bioethanol.

The present disclosure relates to a culture medium comprising tender coconut water, a nitrogen source, and a carbon source, optionally along with magnesium salt.

In an embodiment of the present disclosure, the culture medium comprises a nitrogen source at a concentration of 25mM to 50mM, and a carbon source at a concentration of 0 to 10% (v/v).

In another embodiment of the present disclosure, the culture medium comprises tender coconut water at a percentage of 25% to 100% (v/v).

In yet another embodiment of the present disclosure, the carbon source is selected from a group comprising, glycerol, glucose, fructose and sucrose or any combination thereof.

In yet another embodiment of the present disclosure, the glycerol is at a concentration selected from 0%, 0.1%, 0.5%, 1%, 5%, or 10% (v/v).

In yet another embodiment of the present disclosure, the carbon source selected from the group comprising glucose, fructose and sucrose or any combination thereof, is at a concentration selected from 0%, 0.1%, 0.5%, 1%, 5%, or 10% (w/v).

In yet another embodiment of the present disclosure, the nitrogen source is selected from a group comprising ammonium sulphate, ammonium acetate, ammonium carbonate, ammonium chloride and amino acid mix or any combination thereof.

In still another embodiment of the present disclosure, the culture medium comprises tender coconut water at a concentration of 25mM ammonium sulphate, glucose at 1% (w/v), sucrose at 1% (w/v), glycerol at 1.25% (v/v) from 80% (v/v) of stock solution, and fructose at 1% (w/v).

In still another embodiment of the present disclosure, the culture medium comprises tender coconut water at a concentration of 25mM ammonium sulphate, glucose at 1% (w/v), sucrose at 1% (w/v), glycerol at 2.5% (v/v) from 80% (v/v) of stock solution, and fructose at 1% (w/v).

In still another embodiment of the present disclosure, the culture medium comprises magnesium salt at a concentration of 2mM, wherein the magnesium salt is selected from a group comprising magnesium sulphate, magnesium chloride and magnesium carbonate.

In still another embodiment of the present disclosure, the culture medium enhances the growth of yeast. The fold increase in yeast growth in tender coconut water- based culture medium when compared to the yeast growth in YEPD (yeast extract, peptone, dextrose) medium is found to be 6 times.

According to an embodiment of the present disclosure, the culture medium is prepared by:

a) extracting tender coconut water from tender coconuts;
b) filter sterilizing the extracted tender coconut water;
c) taking the filtered sterilized tender coconut water at percentages ranging from 25% to 100%;
d) adding a nitrogen source at a concentration ranging from 25mM to 50mM; and
e) adding a carbon source at a concentration ranging from 0-10%.

Further, the present disclosure relates to a method of culturing yeast comprising steps of:

a) adding warm water to yeast to obtain activated yeast;
b) serially diluting the activated yeast in saline, wherein the serial dilution factors are 10^{-1} to 10^{-5};
c) plating inoculums from each of the serial dilutions in tender coconut water agar (TCW agar) medium taken in five culture plates respectively labelled from 10^{-1} to 10^{-5}, to obtain TCW agar plates;
d) incubating the TCW agar plates at 30°C for 48 hours to obtain yeast colonies on the plates;
e) inoculating a single colony from the10^{-3} dilution TCW agar plate into a medium comprising tender coconut water and ammonium sulphate at a concentration of 25 mM to obtain a pre-inoculum;
f) incubating the pre-inoculum at 30°C at 200 rpm for 24 hours;
g) subculturing 1% (v/v) of the pre inoculum in 50 ml of a culture medium comprising tender coconut water, a nitrogen source, and a carbon source, optionally along with magnesium salt, at 30°C for 24 hours to obtain subcultures of the yeast ; and
h) harvesting cells from the subcultures to obtain the yeast.

In an embodiment of the present disclosure, the TCW agar medium comprises tender coconut water and 4% of agar, wherein the tender coconut water and agar are at a ratio of 1:1.

Furthermore, the present disclosure relates to a method for producing ethanol from an inoculum of yeast cultured in a culture medium comprising tender coconut water, a nitrogen source, and a carbon source, optionally along with magnesium salt comprising steps of:

a) inoculating 20% (v/v) of inoculum of yeast in 100 ml of the medium as claimed in claim 1 to obtain the inoculated medium;
b) incubating the inoculated medium at 30°C at 200 rpm for an incubation period ranging from 4 to 16 hours to get samples;
c) collecting samples after incubation;
d) centrifuging the samples at 4500 rpm, at 4°C, for 40minutes;
e) obtaining cell-free supernatent comprising ethanol; and
f) storing the cell-free supernatent at -20°C.

In an embodiment of the present invention, feasibility of using tender coconut water-based ethanol production, which is clean and pharmaceutical grade is described. Yeast inoculum size has a significant effect on ethanol production (Turhan et al. 2010). In an aspect of the present invention different inoculum sizes, i.e., 1%, 5%, 10% and 20% (v/v) are used, and it was observed that the amount of ethanol produced gradually increased with the increase in inoculum size.

These and other aspects of the embodiments herein will be better appreciated and understood when considered in conjunction with the following description and the accompanying drawings. It should be understood, however, that the following descriptions, while indicating preferred embodiments and numerous specific details thereof, are given by way of illustration and not of limitation. Many changes and modifications may be made within the scope of the embodiments herein without departing from the scope thereof, and the embodiments herein include all such modifications.

BRIEF DESCRIPTION OF DRAWINGS

FIG.1 illustrates the optical densities recorded for the combination A comprising sub-sets A1 to A4.

FIG. 2 illustrates the optical densities recorded for the combination B comprising sub-sets B1 to B4.

FIG. 3 illustrates the optical densities recorded for the combination C comprising sub-sets C1 to C4.

FIG. 4 illustrates the optical densities recorded for the combination D comprising sub-sets D1 to D4.

FIG. 5 illustrates the optical densities recorded for the combination E comprising sub-sets E1 to E4.

FIG. 6 illustrates the optical densities recorded for the combination F comprising sub-sets F1 to F4.

FIG. 7 illustrates the optical densities recorded for the combination G comprising sub-sets G1 to G4.

FIG. 8 illustrates the optical densities recorded for the combination H comprising sub-sets H1 to H4.

FIG. 9 illustrates the optical densities recorded for the combination I comprising sub-sets I1 to I4.

FIG. 10 illustrates a comparison of the growth of yeast in the present Tender coconut water-based media versus growth of yeast in YEPD (yeast extract, peptone, dextrose) media and Molasses.

FIG. 11 illustrates the optical densities recorded for the combination (i) comprising 6 sub-sets for 0, 0.1%, 0.5%, 1.0%, 5.0% and 10.0 % (v/v) glycerol with 25mM Ammonium sulphate.

FIG. 12 illustrates the optical densities recorded for the combination (ii) comprising 6 sub-sets for 0, 0.1%, 0.5%, 1.0%, 5.0% and 10.0 % (v/v) glycerol with 25mM Ammonium sulphate and 2mM Magnesium sulphate.

FIG. 13 illustrates the optical densities recorded for the combination (iii) comprising 6 sub-sets for 0, 0.1%, 0.5%, 1.0%, 5.0% and 10.0 % (v/v) glycerol with 50mM Ammonium sulphate.

FIG. 14 illustrates the optical densities recorded for the combination (iv) comprising 6 sub-sets for 0, 0.1%, 0.5%, 1.0%, 5.0% and 10.0 % (v/v) glycerol with 50mM Ammonium sulphate and 2mM Magnesium sulphate.

FIG. 15 illustrates a comparison of the growth of baker's yeast in the present Tender coconut water- based media versus growth of yeast in YEPD (yeast extract, peptone, dextrose) media, performed at a large scale (i.e. 100 ml culture in 500 ml flask).

FIG. 16 illustrates sensory analysis of bread prepared using baker's yeast grown in optimised TCW media and YPD media.

FIG. 17 illustrates GC-MS data for determination of ethanol.

FIG. 18 illustrates the production of ethanol at periodic intervals of time (at 0^{th}, 4^{th}, 8^{th}, 12^{th} and 16^{th} hour) for yeast inoculum size of 1%, 5%, 10%, and 20% (v/v).

FIG. 19a illustrates a bar-chart indicating the fold changes occurring at 16 hours with the sub sets of the combinations A to I.

FIG. 19b illustrates a bar-chart indicating the fold changes occurring at 20 hours with the sub sets of the combinations A to I.

FIG. 19c illustrates a bar-chart indicating the fold changes occurring at 24 hours with the sub sets of the combinations A to I.

FIG. 19d illustrates a bar-chart indicating the fold changes occurring at 28 hours with the sub sets of the combinations A to I.

FIG. 19e illustrates a radar chart plotted by merging the fold change values for the subsets of the combinations A to I, with the time points (i.e. 16, 20, 24, and 28 hours) together.

DETAILED DESCRIPTION OF THE INVENTION

The following description with reference to the accompanying drawings is provided to assist in a comprehensive understanding of exemplary embodiments of the invention. It includes various specific details to assist in that understanding but these are to be regarded as merely exemplary.

The primary objective of the present invention is to produce an organic media, particularly a tender coconut water-based medium.

Another objective of the present invention is to culture high quantity of healthy yeast cells in an organic way (free from animal-based media or vegan method) using tender coconut water-based medium. The organic tender coconut water-based culture medium of the present disclosure comprises sugars which are nutritious for yeast. It has been investigated in the present disclosure how carbon sources or sugars, when provided at the right combination, amount or at the optimum concentration in the culture medium, result in better and faster growth of yeast cells.

Another objective of the present invention is to prepare bread using yeast cultured in an organic way.

Yet another objective of the present invention is to produce ethanol using the tender coconut water-based medium.

The present disclosure relates to a culture medium comprising tender coconut water, a nitrogen source, and a carbon source, optionally along with magnesium salt.

In an embodiment of the present disclosure, the culture medium comprises a nitrogen source at a concentration of 25mM to 50mM, and a carbon source at a concentration of 0 to 10% (v/v).

In another embodiment of the present disclosure, the culture medium comprises tender coconut water at a percentage of 25% to 100% (v/v).

In yet another embodiment of the present disclosure, the carbon source is selected from a group comprising, glycerol, glucose, fructose and sucrose or any combination thereof.

In yet another embodiment of the present disclosure, the glycerol is at a concentration selected from 0%, 0.1%, 0.5%, 1%, 5%, or 10% (v/v).

In yet another embodiment of the present disclosure, the carbon source selected from the group comprising glucose, fructose and sucrose or any combination thereof, is at a concentration selected from 0%, 0.1%, 0.5%, 1%, 5%, or 10% (w/v).

In yet another embodiment of the present disclosure, the nitrogen source is selected from a group comprising ammonium sulphate, ammonium acetate, ammonium carbonate, ammonium chloride and amino acid mix or any combination thereof.

In still another embodiment of the present disclosure, the culture medium comprises tender coconut water at a concentration of 25mM ammonium sulphate, glucose at 1% (w/v), sucrose at 1% (w/v), glycerol at 1.25% (v/v) from 80% (v/v) of stock solution, and fructose at 1% (w/v).

In still another embodiment of the present disclosure, the culture medium comprises tender coconut water at a concentration of 25mM ammonium sulphate, glucose at 1% (w/v), sucrose at 1% (w/v), glycerol at 2.5% (v/v) from 80% (v/v) of stock solution, and fructose at 1% (w/v).

In still another embodiment of the present disclosure, the culture medium comprises magnesium salt at a concentration of 2mM, wherein the magnesium salt is selected from a group comprising magnesium sulphate, magnesium chloride and magnesium carbonate.

In still another embodiment of the present disclosure, the culture medium enhances the growth of yeast.

According to an embodiment of the present disclosure, the culture medium is prepared by:

a) extracting tender coconut water from tender coconuts;

b) filter sterilizing the extracted tender coconut water;

c) taking the filtered sterilized tender coconut water at percentages ranging from 25% to 100%;

d) adding a nitrogen source at a concentration ranging from 25mM to 50mM; and

e) adding a carbon source at a concentration ranging from 0-10%.

In an embodiment, at step b), filter sterilizing the extracted tender coconut water is performed using 11µm Whatman filter paper followed by 0.22µm PVDF Merck Millipore filter membrane; or any sequential filtration process can be followed for reducing the cost involved in the use of 0.22 µm PVDF.

Further, the present disclosure relates to a method of culturing yeast comprising steps of:

a) adding warm water to yeast to obtain activated yeast;

b) serially diluting the activated yeast in saline, wherein the serial dilution factors are 10^{-1} to 10^{-5};

c) plating inoculums from each of the serial dilutions in tender coconut water agar (TCW agar) medium taken in five culture plates respectively labelled from 10^{-1} to 10^{-5} , to obtain TCW agar plates;

d) incubating the TCW agar plates at 30°C for 48 hours to obtain yeast colonies on the plates;

e) inoculating a single colony from the10^{-3} dilution TCW agar plate into a medium comprising tender coconut water and ammonium sulphate at a concentration of 25 mM to obtain a pre-inoculum;

f) incubating the pre-inoculum at 30°C at 200 rpm for 24 hours;

g) sub-culturing 1% (v/v) of the pre-inoculum in 50 ml of a culture medium comprising tender coconut water, a nitrogen source, and a carbon source, optionally along with magnesium salt, at 30°C for 24 hours to obtain subcultures of the yeast; and

h) harvesting cells from the subcultures to obtain the yeast.

In an embodiment, at the step g) the optical density of the subcultures is recorded at 600nm using spectrophotometer.

In an embodiment of the present disclosure, the TCW agar medium comprises tender coconut water and 4% of agar, wherein the tender coconut water and agar are at a ratio of 1:1.

Furthermore, the present disclosure relates to a method for producing ethanol from an inoculum of yeast cultured in a culture medium comprising tender coconut water, a nitrogen source, and a carbon source, optionally along with magnesium salt comprising steps of:

a) inoculating 20% (v/v) of inoculum of yeast in 100 ml of the medium as claimed in claim 1 to obtain the inoculated medium;

b) incubating the inoculated medium at 30°C at 200 rpm for an incubation period ranging from 4 to 16 hours to get samples;

c) collecting samples after incubation;

d) centrifuging the samples at 4500 rpm, at 4°C, for 40minutes;

e) obtaining cell-free supernatant comprising ethanol; and

f) storing the cell-free supernatant at -20°C.

In an embodiment, the cell free supernatant collected at step f) was used for further studies.

In another embodiment, the cell free supernatant collected at step f) was subjected to gas chromatography (GC) to determine the ethanol content. The supernatant was collected and added with 1% internal standard (ethanol) and injected into the GC.

EXAMPLES:

Example 1: Culturing of yeast in growth media comprising tender coconut water, nitrogen source and various combinations of carbon sources.

Tender coconut water (TCW) contains reducing sugars like fructose and glucose, and non-reducing sugars like sucrose. To optimize the combinations for improved yeast growth, the quantities of TCW used in the combinations are minimized, diluted with water and additionally carbon sources are added. Since simple sugars like sucrose, fructose, and glucose (w/v) are included in addition to the naturally present carbon sources in TCW, this ensures the availability of adequate nutrients for the growth of the yeast. Here, nine types of the media are prepared with different ratios of tender coconut water (for example, 25%, 50%, 75% and 100% (v/v)) to which sugars like glucose, sucrose and fructose are added in various combinations. In all these combinations ammonium sulphate is employed as the source of nitrogen. The purpose of testing of various combinations to check for yeast growth, is to identify the best combination that yields more biomass of yeast and reduces the cost of the growth media.

Preparation of reagents:

1. **<u>Tender Coconut Water:</u>** Tender coconuts are procured from farm/vendor (for the present experiment, the tender coconuts were purchased from a street-side vendor in Bengaluru, India). The fruits are cut open to extract the water. The extracted tender

coconut water (TCW) is filter sterilized using 11µm Whatman filter paper followed by 0.22µm PVDF Merck Millipore filter membrane. The two-membrane system is used to avoid clogging of the 0.22µm PVDF membrane hence, reduce frequent change of this membrane. The filtered TCW is stored at 4°C until further use.

2. **Ammonium sulphate:** 1M ammonium sulphate is prepared as a stock solution by weighing 132.14 g of ammonium sulphate and dissolving it in 1000ml of distilled/MilliQ water.

3. **Glycerol:** 80% (v/v) glycerol is prepared by taking 80 ml of 100% glycerol and adding into 20 ml of distilled/MilliQ water. It is autoclaved at 120°C, 15psi, for 30 minutes.

4. **Molasses:** 4 ml of molasses is taken, diluted with 96 ml of distilled/MilliQ water and autoclaved.

5. **Other chemicals or reagents:** High purity fructose, glucose, and sucrose were sterilized with 0.22µm PVDF filtration prior to use.

The above reagents are made for generating the combinations labelled A, B, C, D, E, F, G, H and I (see Table 1 below). In these combinations, there are sub-sets prepared with an intention to optimize the TCW usage. For example, sub-sets labelled as 1, 2, 3, 4 are prepared for each of the combinations (A-I). The subsets 1, 2, 3, and 4 represent 25%, 50%, 75% and 100% of TCW respectively. The efficiency of the media composition(s) was calculated by difference in the optical density (OD) between 'test' and 'control' (I4). Here, 'test' refers to the combinations of the media and the 'control' is TCW (100%) with 25mM ammonium sulphate. Fold change is calculated using formula: Test / Control (I4) = Fold(s).

Procedure for culturing yeast:

Nine combinations (A to I) have been tested for growth of yeast. The steps for culturing yeast in these combinations are as follows:

1. Yeast (*Saccharomyces cerevisiae*) from a commercially available source Moments ™ is taken for preparing glycerol stock. For preparing the glycerol stock of yeast, firstly yeast is activated by the addition of 1 ml of warm water (40-45°C) to 1.5 ml micro centrifuge tube with a pinch of dry yeast from the package followed by swirling the tube upside down 3-4 times. Next, the activated yeast is serially diluted from 10^{-1} to 10^{-5}. The process of serial dilution involves taking five 1.5ml tubes and labelling the tubes from 10^{-1} to 10^{-5}, adding 900 µl of culture media to all tubes, followed by adding 100 µl from the activated yeast tube to the first tube (i.e. labelled as 10^{-1} dilution) and mixing the contents. Further, 100 µl from the first tube is added to the second tube (i.e. labelled as 10^{-2} dilution) and the contents of the second tube are mixed. This process is repeated for the remaining tubes. 100 µl from each of the five dilutions are plated on YEPDA (yeast extract peptone dextrose agar) taken in five plates labelled with the respective dilution factor. These plates were incubated at 30° C incubator for 48 hours. Yeast colonies were observed on the plates. A single colony from the 10^{-3} dilution plate was inoculated into 5 ml of YEPD and incubated overnight, at 30° C, 200 rpm, and then in a cryotube 500 µl of 30% (v/v) glycerol is added to 500 µl of yeast cells followed by

immediate freezing using liquid nitrogen. These are thereafter stored at -80 °C at to obtain glycerol stock.

A loopful of yeast from the glycerol stock culture is inoculated in 10 ml of TCW comprising 25mM Ammonium sulphate (i.e, 250µL from 1M stock solution) and incubated for 48 hours at 30°C, with shaking at the speed of 200 RPM.

2. Tender coconut water is taken in four different dilutions (25%, 50%, 75%, and 100%).
3. The other components are added as mentioned in the table (Table 1) except sterile glycerol to obtain mixtures at various combinations and dilutions.
4. The mixtures prepared in the step 3 are filtered through 0.22µm filter and the filtrates are labelled respectively (all the steps were performed inside Laminar air flow). The filtrates are taken in well plates (a 24 well plate format is used for the experiment) Thereafter, the glycerol is added to the respective wells individually.
5. 1% of the yeast inoculum is sub cultured into the combinations (A-I). The combinations (A-I) are depicted in the Table 1.
6. The 24 well plate is incubated at 30°C, with shaking at a speed of 200 RPM.
7. The Optical density (OD) is measured at 600mm on the 16th, 20th, 24th and 28th hour. A standard graph of OD versus Time is plotted (Fig. 1 to 9). It is observed that the maximum optical density of cells is usually obtained between 20- 28 hours as per the repeated and routine of the experiments with TCW.

Table 1: Combinations and dilutions (sub-sets) of the growth media (A-I):

Combination A				
TCW	2.5 ml (A1)	5 ml (A2)	7.5 ml (A3)	9.6 ml (A4)
Glucose	100 mg	100 mg	100 mg	100 mg
Sucrose	100 mg	100 mg	100 mg	100 mg
Glycerol (80%)	125 µl	125 µl	125 µl	125 µl
Fructose	100 mg	100 mg	100 mg	100 mg
Water	Make up to 10 ml			
Ammonium Sulphate	25mM (i.e 250 µl from 1M stock)			
Combination B				
TCW	2.5 ml (B1)	5 ml (B2)	7.5 ml (B3)	9.6 ml (B4)
Glucose	200 mg	200 mg	200 mg	200 mg
Sucrose	100 mg	100 mg	100 mg	100 mg
Glycerol (80%)	125 µl	125 µl	125 µl	125 µl
Fructose	NA			
Water	Make up to 10 ml			

Ammonium Sulphate	25mM (i.e 250 µl from 1M stock)			
Combination C				
TCW	2.5 ml (C1)	5 ml (C2)	7.5 ml (C3)	9.6 ml (C4)
Glucose	200 mg	200 mg	200 mg	200 mg
Sucrose	200 mg	200 mg	200 mg	200 mg
Glycerol (80%)	NA			
Fructose	NA			
Water	Make up to 10 ml			
Ammonium Sulphate	25mM (i.e 250 µl from 1M stock)			
Combination D				
TCW	2.5 ml (D1)	5 ml (D2)	7.5 ml (D3)	9.6 ml (D4)
Glucose	200 mg	200 mg	200 mg	200 mg
Sucrose	NA			
Glycerol (80%)	250 µl	250 µl	250 µl	250 µl
Fructose	NA			
Water	Make up to 10 ml			
Ammonium Sulphate	25mM (i.e 250 µl from 1M stock)			
Combination E				
TCW	2.5 ml (E1)	5 ml (E2)	7.5 ml (E3)	9.6 ml (E4)
Glucose	NA			
Sucrose	200 mg	200 mg	200 mg	200 mg
Glycerol (80%)	250 µl	250 µl	250 µl	250 µl
Fructose	NA			
Water	Make up to 10 ml			
Ammonium Sulphate	25mM (i.e 250 µl from 1M stock)			
Combination F				
TCW	2.5 ml (F1)	5 ml (F2)	7.5 ml (F3)	9.6 ml (F4)
Glucose	NA			
Sucrose	400 mg	400 mg	400 mg	400 mg
Glycerol (80%)	NA			
Fructose	NA			
Water	Make up to 10 ml			
Ammonium Sulphate	25mM (i.e 250 µl from 1M stock)			

Combination G				
TCW	2.5 ml (G1)	5 ml (G2)	7.5 ml (G3)	9.6 ml (G4)
Glucose	NA			
Sucrose	NA			
Glycerol (80%)	NA			
Fructose	400 mg	400 mg	400 mg	400 mg
Water	Make up to 10 ml			
Ammonium Sulphate	25mM (i.e 250 µl from 1M stock)			
Combination H				
TCW	2.5 ml (H1)	5 ml (H2)	7.5 ml (H3)	9.6 ml (H4)
Glucose	NA			
Sucrose	NA			
Glycerol (80%)	500 µl	500 µl	500 µl	500 µl
Fructose	NA			
Water	Make up to 10 ml			
Ammonium Sulphate	25mM (i.e 250 µl from 1M stock)			
Combination I				
TCW	2.5 ml (**I**1)	5 ml (**I**2)	7.5 ml (**I**3)	9.6 ml (**I**4)
Glucose	NA			
Sucrose	NA			
Glycerol (80%)	NA			
Fructose	NA			
Water	Make up to 10 ml			
Ammonium Sulphate	25mM (i.e 250 µl from 1M stock)			

Results and Discussions:

In this experiment, different culture media combinations with tender coconut water were tested to know the best suitable combination for growth of yeast (*Saccharomyces cerevisiae*). Multiple combinations (A to I) were prepared with tender coconut water, glucose, fructose, sucrose and glycerol to identify which of the combinations and components play a vital role in growth of yeast. Each combination contains 4 sub-sets marked as 1, 2, 3, 4 (referring to Table 1) wherein each sub-set differ with respect to the tender coconut water ratio (25%, 50%, 75%, 100% (v/v)). Yeast is cultured in the said multiple combinations. The optical density data of these combinations are compared to that of the 'control' - a combination (I4) comprising 100% (v/v) tender coconut water containing 25mM ammonium sulphate. This is to know if these combinations can match or can be more efficient than the 'control'. The sets (A1, A2, A3 and

A4) in combination A containing all the components i.e., glucose, fructose, sucrose and glycerol significantly show higher growth than the other sets of combinations.

The maximum fold change obtained was 1.835 and 1.787 times at 28th and 24th in A3 respectively when compared to the control I4. The maximum observed OD in control (I4) is 3.9 at 28th hour which is lesser than the OD obtained in combination A2, A3, A4, B3, B4. C3, C4, D3, D4, E2, E3, and E4 (Table 2). This is due to the effect of additional components. The best combination found is 6.923 in A3 combination that contains only 75% tender coconut water with all other components. The A3 set in combination A contains 75% tender coconut water with glucose, sucrose, glycerol, fructose and 25mM ammonium sulphate. The yeast utilizes these components which is present in the combination A and is found to show better yield than any of the other combinations.

Table 2: Optical densities of the combinations and dilutions (A-I):

	Hours	**16**	**20**	**24**	**28**
Optical Density (600mm)	**A1**	2.140	2.620	3.413	3.687
	A2	2.203	4.987	6.140	6.700
	A3	2.970	6.923	7.007	7.193
	A4	2.623	6.140	6.438	6.669
	B1	2.332	2.192	2.147	2.193
	B2	2.500	3.420	3.760	3.865
	B3	3.036	4.390	4.337	4.216
	B4	3.113	4.410	4.817	5.028
	C1	2.440	1.857	2.092	2.118
	C2	3.248	3.592	3.872	4.181
	C3	3.007	4.358	4.490	4.781
	C4	2.887	4.553	4.940	5.250
	D1	2.185	1.950	2.117	2.170
	D2	2.726	3.027	3.703	3.885
	D3	2.492	2.902	4.087	4.210
	D4	2.417	3.310	4.477	4.593
	E1	2.115	1.962	2.647	2.823
	E2	2.757	3.677	4.093	4.640
	E3	2.598	3.447	4.337	4.847
	E4	2.630	3.405	4.500	5.340
	F1	1.553	1.853	2.210	2.730
	F2	2.090	2.405	2.904	3.280
	F3	2.547	2.788	3.308	3.577
	F4	3.097	3.317	3.513	3.893
	G1	1.097	1.115	1.297	1.470
	G2	1.047	1.142	1.279	1.319
	G3	1.447	1.763	1.923	2.226
	G4	1.760	2.297	2.787	3.190

	H1	1.091	1.114	1.133	1.187
	H2	1.113	1.144	1.215	1.330
	H3	1.510	1.893	2.231	2.481
	H4	1.713	2.500	2.907	3.343
	I1	1.310	1.638	2.327	2.922
	I2	1.767	2.117	2.532	3.096
	I3	2.003	2.419	2.843	3.477
	I4	2.397	2.850	3.553	3.920

The top 10-fold change (Table 3) out of 36 sets in combinations A to I occurs in set A3, A4, A2, and C4. This data clearly indicates that the addition of one simple sugar to combination with 25%, 50%, 75% and 100% (v/v) tender coconut water does not support the yeast cells as much as the combination with multiple sugars. This difference is due to fact that sugar is nutritious for yeast. When sugar is provided at the right amount or at the optimum concentration, the cells grow better and faster. However larger concentration of sugar will decline yeast growth and size.

Using Table 3 data, a bar-chart was plotted indicating the fold changes occurring at regular intervals of time i.e. 16, 20, 24, and 28 hours, with the sub sets of the combinations A to I (Fig. 19a to 19d), In the bar chart, the yellow line indicates the border line indicating the fold change is higher than the control if the bar crosses the line. Here, the fold change was calculated by using the maximum OD obtained at control I4 where 100% (v/v) of TCW with 25mM ammonium sulphate was used. Also, a radar chart was plotted by merging the fold change values with all-time points together (Fig. 19e).

Table 3: Fold change of the combinations and dilutions (A-I):

	Hours	**16**	**20**	**24**	**28**
FOLD CHANGE	**A1**	0.893	0.919	0.961	0.940
	A2	0.919	1.750	1.728	1.709
	A3	1.239	2.429	1.972	1.835
	A4	1.095	2.154	1.812	1.701
	B1	0.973	0.769	0.604	0.560
	B2	1.043	1.200	1.058	0.986
	B3	1.267	1.540	1.220	1.075
	B4	1.299	1.547	1.356	1.283
	C1	1.018	0.651	0.589	0.540
	C2	1.355	1.260	1.090	1.067
	C3	1.255	1.529	1.264	1.220
	C4	1.204	1.597	1.390	1.339
	D1	0.912	0.684	0.596	0.554
	D2	1.137	1.062	1.042	0.991
	D3	1.040	1.018	1.150	1.074
	D4	1.008	1.161	1.260	1.172

	E1	0.882	0.688	0.745	0.720
	E2	1.150	1.290	1.152	1.184
	E3	1.084	1.209	1.220	1.236
	E4	1.097	1.195	1.266	1.362
	F1	0.648	0.650	0.622	0.696
	F2	0.872	0.844	0.817	0.837
	F3	1.063	0.978	0.931	0.912
	F4	1.292	1.164	0.989	0.993
	G1	0.458	0.391	0.365	0.375
	G2	0.437	0.401	0.360	0.336
	G3	0.604	0.619	0.541	0.568
	G4	0.734	0.806	0.784	0.814
	H1	0.455	0.391	0.319	0.303
	H2	0.465	0.401	0.342	0.339
	H3	0.630	0.664	0.628	0.633
	H4	0.715	0.877	0.818	0.853
	I1	0.547	0.575	0.655	0.745
	I2	0.737	0.743	0.713	0.790
	I3	0.836	0.849	0.800	0.887
	I4	1.000	1.000	1.000	1.000

In the present disclosure, yeast growth is also compared with commercially available culture media like YEPD (Yeast Extract, Peptone and Dextrose) and molasses. It is found that the control combination (I4) is more efficient and gives more yield (Fig. 10). Large culture volumes (100ml) of 4% (v/v) molasses, YEPD and TCW are prepared. The maximum OD of 26 was seen at 24th hour in TCW with 25mM ammonium sulphate. The procedure for comparison of yeast growth in TCW and YEPD is explained in greater detail in the below example. The present TCW media is found to lower the cost of growth media, as it involves addition of simple sugars, it boosts yield quickly, and, most importantly, facilitates a clean, vegan method of culturing yeast. By employing this media and technique, the quality of baker's yeast's is also improved.

EXAMPLE 2:

Growth comparison of *Saccharomyces cerevisiae* in TCW and YEPD

Materials and Methods

Commercially available dry yeast (Moments ^{TM}), Tender Coconut Water (TCW), Yeast extract, peptone, dextrose, agar, ammonium sulphate and glycerol.

Tender Coconut Water

TCW was supplied by a shop from a local market in Mysore district, Karnataka, India. To prepare a culture medium, the exocarp (outer coat) of coconut was wiped with 70% ethanol, and the TCW was dispensed in a sterile beaker inside the laminar air flow chamber. TCW was then transferred to 50ml sterile falcon tubes and centrifuged at 4000 to 5000 rpm (4-6°C) for

20 to 40 minutes to separate suspended solids. The supernatant was filtered through a 0.22µm filter membrane (Millex filter units- Millipore) and stored at 4°C until use.

YEPD, YEPDA and TCW-Agar media

Yeast extract, peptone, and dextrose (YEPD) and agar (YEPDA) media are commonly used for culturing yeast. The ingredients of YEPDA are 1% (w/v) yeast extract, 2% (w/v) Peptone, 2% (w/v) Dextrose and 2% (w/v) agar. Here, this media is used for comparison with TCW agar (TCW-agar) media. TCW-A is prepared by take 4% of agar and adding it into TCW, wherein the agar and TCW are at a ratio of 1:1, and pouring this mixture into petri dish.

Starter culture preparation

Saccharomyces cerevisiae was activated by the addition of 1 ml of warm water (40-45°C) to 1.5 ml microcentrifuge tube with a pinch of dry yeast followed by swirling the tube upside down 3-4 times. Next, the activated yeast is serially diluted from 10^{-1} to 10^{-5} and plated on TCW-agar and YEPDA. The process of serial dilution involves taking five 1.5ml tubes and labelling the tubes from 10 -1 to 10 -5, adding 900 µl of media to all tubes, followed by adding 100 µl of activated yeast tube to the first tube (i.e. labelled as 10^{-1} dilution) and mixing the contents. Further, 100 µl of inoculum from the first tube is added to the second tube (i.e. labelled as 10^{-2} dilution) and the contents of the second tube are mixed. This process is repeated for the remaining tubes. The inoculum from each of the five dilutions are plated on TCW agar taken in five plates labelled with the respective dilution factor and also in YEPDA taken in five plates labelled with the respective dilution factor. These plates were incubated at 30°C incubator for 48 hours. Yeast colonies were observed on both the TCW-A as well as YEPDA plates.

Additional nutrients

The growth of yeast in TCW was increased by the addition of supplementary sources, viz., ammonium sulphate (25Mm) and sterile glycerol (0.1%) as a source of nitrogen and carbon, respectively. 1M of ammonium sulphate was used as stock. This was prepared by adding 13.2g of ammonium sulphate in 100ml of MilliQ water/distilled water. From this stock solution 250 µl is used for supplementing 10ml of TCW. Glycerol is prepared as 10% stock solution, i.e. by mixing 10ml of glycerol in 90ml water. For 5ml of TCW, 10 µl of this glycerol is added as a supplement.

Media optimization, culture conditions, and growth parameters

A single colony from the TCW-agar plate labelled with the dilution factor 10^{-3} was inoculated into of 10mL filter-sterilized TCW having 25mM ammonium sulphate only to obtain a pre-inoculum. Similarly, a colony from the YEPDA plate labelled with the dilution factor 10^{-3} was inoculated into 10mL YEPD. These two pre-inoculums were incubated overnight at 30°C at 200 rpm. The 1% pre-inoculum was sub cultured into a 250 ml conical flask with a working volume of 50 ml TCW with supplements and into 50 ml YEPD. Different combinations of supplements for TCW media, viz., ammonium sulphate and glycerol are mentioned in Table 2. Optical density at 600nm (OD 600nm) was measured every 8 hours by using a spectrophotometer, and a graph was plotted for growth comparison between the TCW and YEPD media. The observations are summarized under the section 'Results' below. The supplemented TCW media with the best condition for high biomass production of yeast was chosen for further experiments.

EXAMPLE 3:

Production of baker's yeast

Production of organic yeast was done by sub culturing (1%) of the inoculum into 2000 ml conical flask containing 500ml TCW supplemented with an optimized condition of 25mM ammonium sulphate and 0.1% glycerol (in triplicate). The said three flasks(triplicates) were incubated at 30° C with 200 rpm. Samples were taken and measured for optical density at OD_{600nm} (JASCO V-630 BIO) at every 6-hour interval. The maximum OD_{600nm} of 33.95 was obtained at 135th hour. Cells were harvested at this point by centrifugation at 4000 rpm, 4° C for 20 minutes.

EXAMPLE 4:

Preparation of bread

The harvested cells were dried at 50-55° C and made into fine granules. The loaf volume of bread mainly depends on the quality and activity level of the yeast. Baker's yeast grown in TCW media along with commercially available baker's yeast were used for baking. The formulation used for the preparation of bread is mentioned in Table 1.

Table 4: Bread formulation (for 200g flour)

S.No.	Ingredients	Quantity (in grams)
1	Flour	200
2	Yeast	4
3	Sugar	5
4	Salt	2
5	Fat	2
6	Water	120 ml

The preparation involves the following steps:

Mixing: Flour was added to the Hobart mixer for two minutes. Activated yeast was added, followed by sugar and salt water. According to the consistency to form a dough, the remaining water was added. The mixer was stopped once the dough turned elastic. Dough was mixed thoroughly on a clean floor, then dusted with flour, and finally kept in a bowl for fermentation.

Fermentation 1: The bowl covered with wet cloth was kept inside the fermentation cabinet for 90 minutes at 30°C. Remix: Dough was mixed by hand until the size of the dough reduced to its original size.

Fermentation 2: Again, the bowl was kept inside the fermentation cabinet for 25 minutes. Sheeting and molding: The fermented dough was stretched and compressed into an elongated shape to fit into a baking pan.

Proofing: The dough inside a baking pan was allowed to proof for 55 minutes at 30°C and 80% relative humidity.

Baking: The proofed dough was placed inside the oven and baked for 25 minutes at 220° C.

Cooling: After baking, the bread was allowed to cool at room temperature for 2 hours and then the bread was packed in a polythene bag.

EXAMPLE 5

Production of ethanol

For ethanol production, four 250 ml flasks with a working volume of 100 ml TCW (supplemented with 25m M ammonium sulphate and 0.1% glycerol) were used. 1%, 5%, 10% and 20% (v/v) of the pre-inoculum (i.e. the filter-sterilized TCW to which a single colony from TCW-A plate was inoculated, mentioned above) was added to the respective conical flasks, and incubation was carried at 30° C with 200 rpm (in duplicate). Samples were collected every 4 hours, centrifuged and the supernatant (top layer) devoid of cells (stored at -20°C until use) was used to determine the ethanol by gas chromatography (Shimadzu GC-2010) and gas chromatography-mass spectrometry (Shimadzu). GC-MS is the gold standard for the confirmation of ethanol. Ethanol produced using TCW media was confirmed by GC-MS (Fig. 17).

GC-MS showed 100% abundance of base peak at 31 m/z (CH_2OH^+) along with peak at 45 corresponding to $CH_3CH_2O^+$ and molecular ion peak ethanol at 46 ($C_2H_5OH^+$). No other major peak was observed which confirms sample has only ethanol as a major by product (Fig. 17). The culture was grown till 16 hours for ethanol production. Samples were collected every 4 hours, centrifuged and the supernatant with internal standard (IS) was injected into gas chromatography (GC). Quantity of the ethanol was estimated by a standard curve drawn using known increasing concentration of EtOH (0.5%, 1%, 1.5%, 2% and 2.5%) with IS. Standard curve was drawn using ratio of ethanol area to IS area. Ethanol was quantified and found to be 20g/L by calculating the ratio of sample to the straight-line equation.

Sample preparation for ethanol analysis

Samples collected every 4-hour were analyzed using gas chromatography (GC), and Carbowax column and flame ionization detector (FID) was used in GC and GCMS. The chromatogram was run at 180°C and 90°C injection temperature using nitrogen as a carrier gas and hydrogen as a flaming gas.

Standard preparation: Analytical grade (AR) ethanol was used at an increasing concentration from 0.5%, 1%, 1.5% to 2% with 1% (v/v) of butanol as an internal standard.

Test preparation: 200µl of cell-free samples were taken with 1% (v/v) of an internal standard. All the test samples and standards were injected using GC syringe (Hamilton) and the data was obtained by calculating the retention time. These selected samples were injected into GCMS for ethanol confirmation.

RESULTS:

In the present invention, optimized conditions for the growth of baker's yeast (*Saccharomyces cerevisiae*) in TCW is achieved. It was followed by growth comparison of *Saccharomyces cerevisiae* in TCW and YEPD, where it is observed that the *Saccharomyces cerevisiae* grown in TCW shows higher biomass than YEPD (Figure 15).

The OD of YEPD was found to be 3.05 at 36^{th} hour and the OD of TCW was found to be 18.9 at 48^{th} hour. Hence, the fold increase in yeast growth in TCW based culture medium when compared to the yeast growth in YEPD is 6 times.

TCW media* optimization *for growth of Saccharomyces cerevisiae

TCW is a naturally available media that is also considered as a vegan media - free from animal sources like beef extract and peptone. Peptones are generally prepared from animal sources (Jayathilakan et al. 2012). TCW is rich in nutrients that support the growth of microbes. In an embodiment of the present invention the TCW media is optimized with the carbon and nitrogen sources for *S. cerevisiae* as listed in Table 5 with a combination of A, B, C and D.

Table 5: TCW- Optimization with various nitrogen (25mM and 50mM ammonium sulphate), carbon (0.1, 0.5, 1.0, 5.0 and 10.0 % (v/v) glycerol) source and 2mM magnesium sulphate

Combination	Ammonium sulphate(mM)	Glycerol (%)	Magnesium sulphate(mM)
(i)	25	0	-
		0.1	
		0.5	
		1	
		5	
		10	
(ii)	25	0	2
		0.1	
		0.5	
		1	
		5	
		10	
(iii)	50	0	-
		0.1	
		0.5	

		1	
		5	
		10	
(iv)	50	0	2
		0.1	
		0.5	
		1	
		5	
		10	

The maximum growth was noticed in the combination (i) (OD_{600nm} 20.3 at 48th hour) at 25mM ammonium sulphate and 0.5% glycerol and also in combination (i), an OD $_{600nm}$ of 18.9 was observed at 25mM ammonium sulphate and 0.1% glycerol as a nitrogen and carbon source respectively (Fig. 11). In combination (ii) having 25mM ammonium sulphate and 0.1% glycerol with 2mM magnesium sulphate, the highest achieved was OD_{600nm} 19.9 at 48th hour (Fig.12), whereas in combination (iii), the maximum OD_{600nm} was 19.8 at 44th hour which contains 50mM ammonium sulphate (Fig.13). A value of 19.9 was observed at OD_{600nm} for combination (iv) comprising 50mM ammonium sulphate and 2mM magnesium sulphate (Fig.14). However, in comparison with 0.1% and 0.5% (v/v) glycerol in combination (i), there is a difference of OD_{600nm} 2.4. The use of 0.1% (v/v) glycerol could be reasonable in terms of minimizing the cost. Therefore, combination (i) having 25mM ammonium sulphate and 0.1% (v/v) glycerol was chosen for further experiments.

Comparison of optimized TCW media and YEPD

S. cerevisiae was grown and compared in yeast specific media YEPD and in the TCW with optimized nitrogen (25mM ammonium sulphate) and carbon source (0.1% glycerol). It was observed that the maximum growth in YEPD was OD_{600nm} 3.05 at 36^{th} hour and in TCW, OD_{600nm} 18.9 at 48^{th} hour (Fig.15). This result shows TCW as a better media for higher used for baking were the same in the test and control. The test and control were subjected to sensory evaluation by the Hedonic 5 scale sensory analysis method. This method is a 5-scale sensory method that includes parameters like appearance, aroma, texture, taste and overall acceptance. These parameters will be scored by people while tasting the product. It is a universal method for food sensory analysis. Scores were plotted along with the sensory parameters, i.e., appearance, aroma, texture, taste and overall acceptability - OAA) in Fig.16. The sensory chart shows that the bread prepared in test media has a higher score in all the parameters and scored higher overall acceptability (OAA) as compared with the control.

Ethanol estimation

Samples inoculated with 1% baker's yeast inoculum showed 1.2, 3.2, 9.9, and 16.8 g/L at 4, 8, 12, and 16^{th} hour respectively. As the inoculum size was increased to 5% there was a gradual increase in ethanol production. It was observed 2.3, 5.7, 15.3 and 19.2 g/L ethanol at 4, 8, 12, and 16^{th} hour, respectively. With 10% inoculum size 3.2, 7.6, 19 and 19.8 g/L and with 20% inoculum size 4.7, 10, 19.8 and 19.4g/L at 4, 8, 12, and 16^{th} hour respectively was observed as in Fig. 18 The results obtained above shows that higher inoculum size results in higher ethanol yield, with 20% inoculum size showing maximum ethanol yield. Mostly 10% and 20% of inoculum size at 12^{th} and 16^{th} hour is more efficient and give comparable ethanol yield.

DISCUSSION

Saccharomyces. cerevisiae also known as Baker's yeast, is commonly used for bread and ethanol production. Use of TCW as a vegan and naturally occurring source of media for high biomass production of yeast, followed by use in bread making and ethanol production has been demonstrated in the present disclosure. TCW media supplemented with ammonium sulphate and glycerol as nitrogen and carbon source, respectively, was successfully used for the growth of *Saccharomyces cerevisiae*. TCW supplemented with 25 mM ammonium sulphate and combination of carbon sources (glucose, fructose and sucrose) collectively gave the highest biomass at an economical cost. This composition also gave significantly higher biomass than the commercially available YEPD media and molasses. In the future, other carbon sources can be utilized for higher and faster growth of *S. cerevisiae* in supplemented TCW media. TCW grown yeast yielded bread that is superior in sensory parameters as well as suitable for ethanol production. Finally, it is concluded that TCW can be employed as a natural, inexpensive and efficient growth media for the production of yeast for the preparation of bread and ethanol.

We Claim:

1. A culture medium comprising tender coconut water, a nitrogen source, and a carbon source, optionally along with magnesium salt.

2. The culture medium as claimed in claim 1 comprising a nitrogen source at a concentration of 25mM to 50mM, and a carbon source at a concentration of 0 to 10% (v/v).

3. The culture medium as claimed in claim 1 comprising tender coconut water at a percentage of 25% to 100% (v/v).

4. The culture medium as claimed in claim 1 wherein the carbon source is selected from a group comprising, glycerol, glucose, fructose and sucrose or any combination thereof.

5. The culture medium as claimed in claim 4, wherein the glycerol is at a concentration selected from 0%, 0.1%, 0.5%, 1%, 5%, or 10% (v/v).

6. The culture medium as claimed in claim 4, wherein the carbon source selected from the group comprising glucose, fructose and sucrose or any combination thereof, is at a concentration selected from 0%, 0.1%, 0.5%, 1%, 5%, or 10% (w/v).

7. The culture medium as claimed in claim 1, wherein the nitrogen source is selected from a group comprising ammonium sulphate, ammonium acetate, ammonium carbonate, ammonium chloride and amino acid mix or any combination thereof.

8. The culture medium as claimed in claim 1 comprising tender coconut water at a concentration of 25mM ammonium sulphate, glucose at 1% (w/v), sucrose at 1% (w/v), glycerol at 1.25% (v/v) from 80% (v/v) stock solution, and fructose at 1% (w/v).

9. The culture medium as claimed in claim 1 comprising tender coconut water at a concentration of 25mM ammonium sulphate, glucose at 1% (w/v), sucrose at 1% (w/v), glycerol at 2.5% (v/v) from 80% (v/v) stock solution, and fructose at 1% (w/v).

10. The culture medium as claimed in claim 1, comprising magnesium salt at a concentration of 2mM, wherein the magnesium salt is selected from a group comprising magnesium sulphate, magnesium chloride and magnesium carbonate.

11. The culture medium as claimed in claim 1, wherein the culture medium enhances the growth of yeast.

12. The culture medium as claimed in claim 1, wherein the culture medium is prepared by:
    a) extracting tender coconut water from tender coconuts;
    b) filter sterilizing the extracted tender coconut water;
    c) taking the filtered sterilized tender coconut water at percentages ranging from 25% to 100%;

d) adding a nitrogen source at a concentration ranging from 25mM to 50mM; and
e) adding and a carbon source at a concentration ranging from 0-10%.

13. A method of culturing yeast comprising steps of:
a) adding warm water to yeast to obtain activated yeast;
b) serially diluting the activated yeast in saline, wherein the serial dilution factors are 10^{-1} to 10^{-5};
c) plating inoculums from each of the serial dilutions in tender coconut water agar (TCW agar) medium taken in five culture plates respectively labelled from 10^{-1} to 10^{-5}, to obtain TCW agar plates;
d) incubating the TCW agar plates at 30°C for 48 hours to obtain yeast colonies on the plates;
e) inoculating a single colony from the10^{-3} dilution TCW agar plate into a medium comprising tender coconut water and ammonium sulphate at a concentration of 25 mM to obtain a pre-inoculum;
f) incubating the pre-inoculum at 30°C at 200 rpm for 24 hours;
g) subculturing 1% (v/v) of the pre inoculum in 50 ml of the medium as claimed in claim 1 at 30°C for 24 hours to obtain subcultures of the yeast; and
h) harvesting cells from the subcultures to obtain the yeast.

14. The method as claimed in claim 13, wherein the TCW agar medium comprises tender coconut water and 4% of agar, wherein the tender coconut water and agar are at a ratio of 1:1.

15. A method for producing ethanol from an inoculum of yeast cultured in a medium as claimed in claim 1 comprising steps of:
a) inoculating 20% (v/v) of inoculum of yeast in 100 ml of the medium as claimed in claim 1 to obtain the inoculated medium;
b) incubating the inoculated medium at 30°C at 200 rpm for an incubation period ranging from 4 to 16 hours to get samples;
c) collecting samples after incubation;
centrifuging the samples at 4500 rpm, at 4°C, for 40minutes;
d) obtaining cell-free supernatent comprising ethanol; and
e) storing the cell-free supernatent at -20°C.

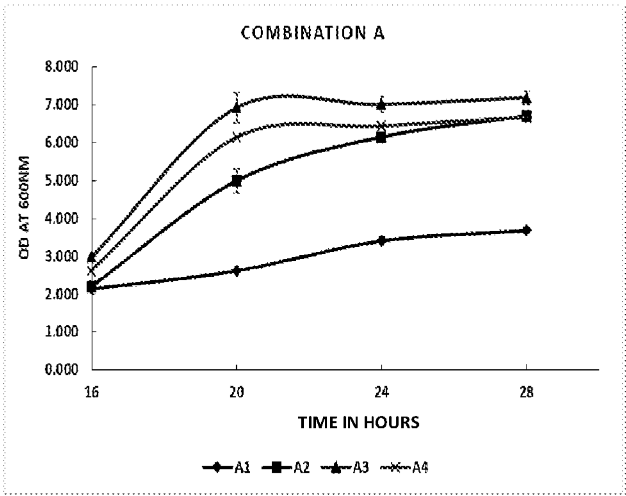

Fig. 1

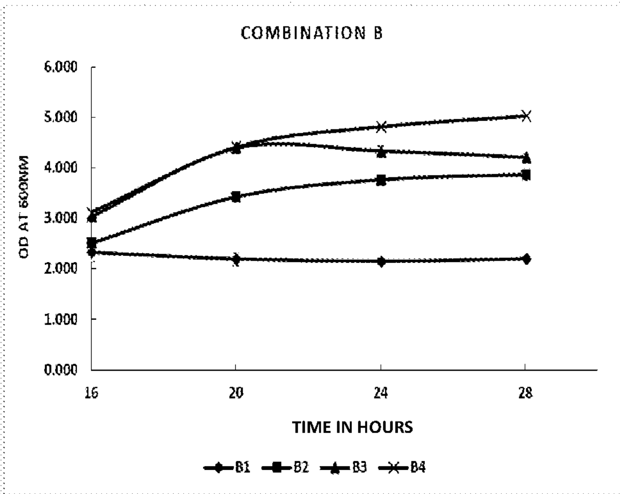

Fig. 2

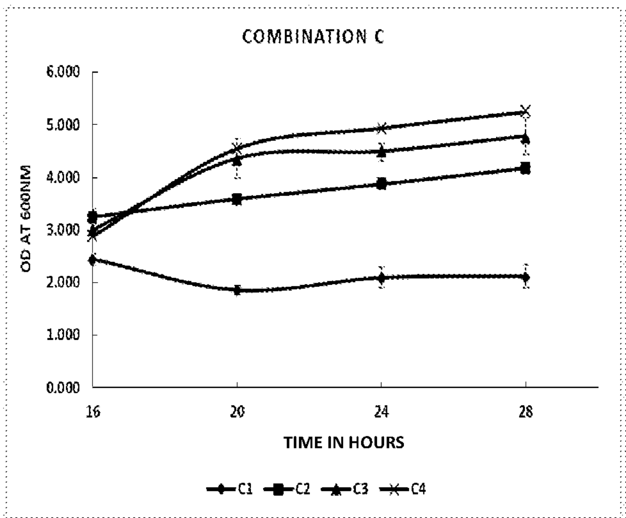

Fig. 3

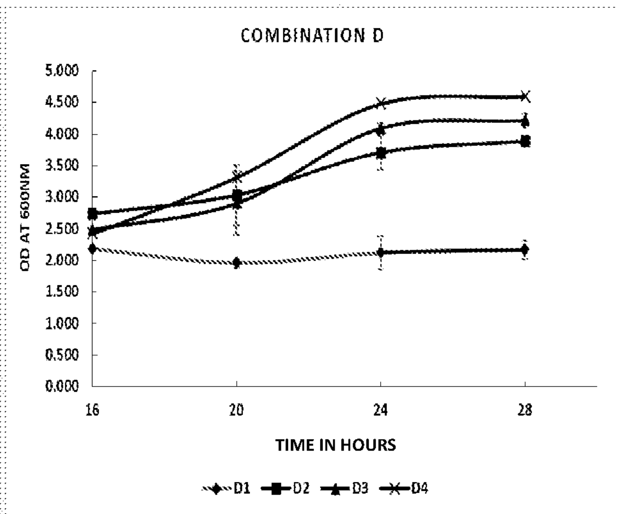

Fig. 4

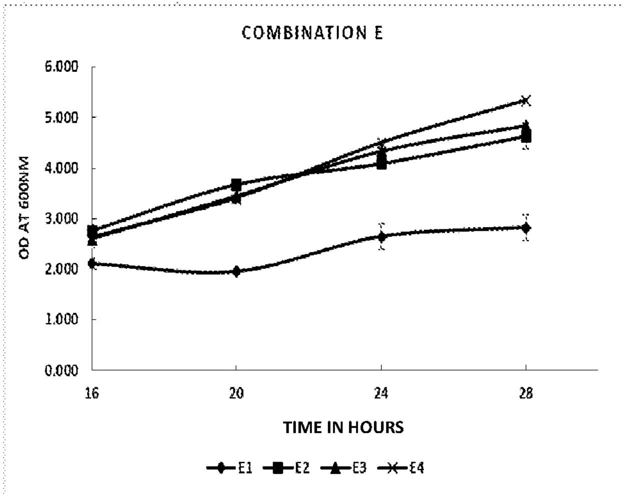

Fig. 5

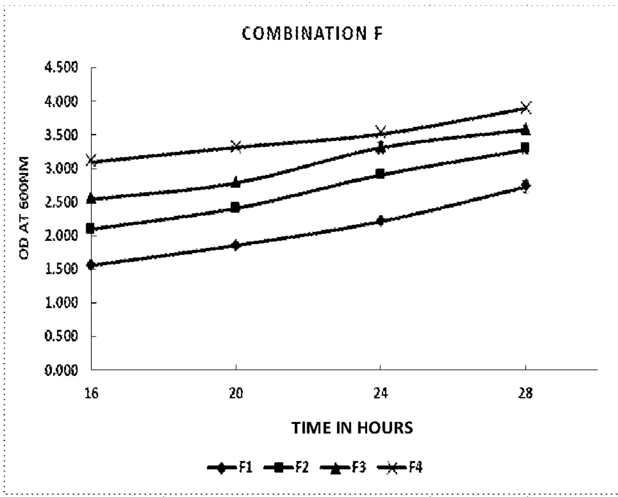

Fig. 6

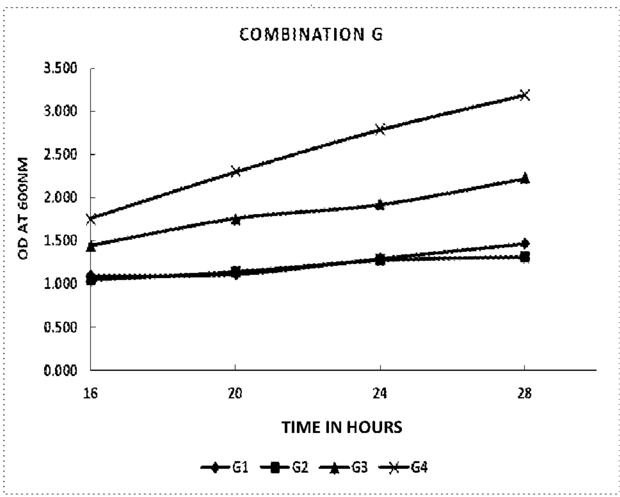

Fig. 7

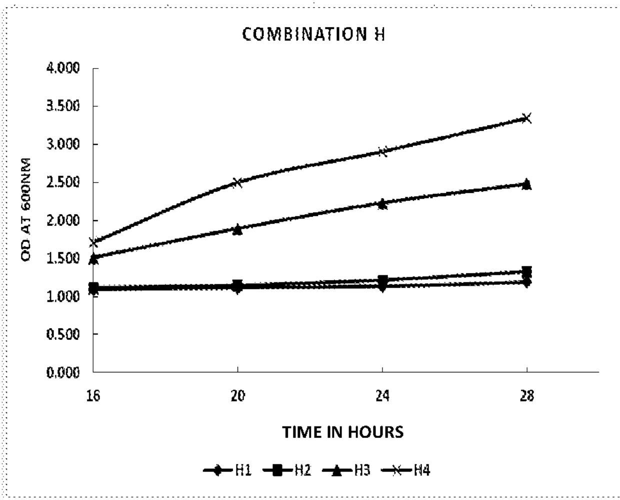

Fig. 8

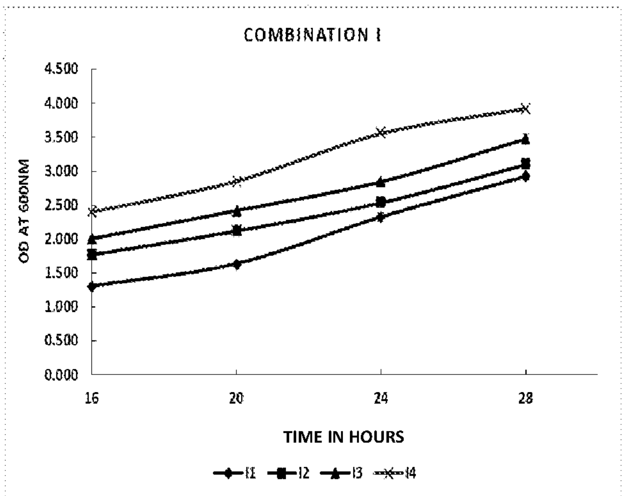

Fig. 9

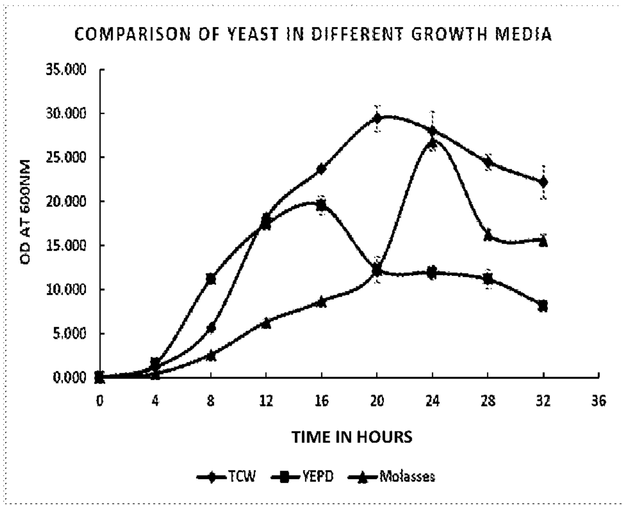

Fig. 10

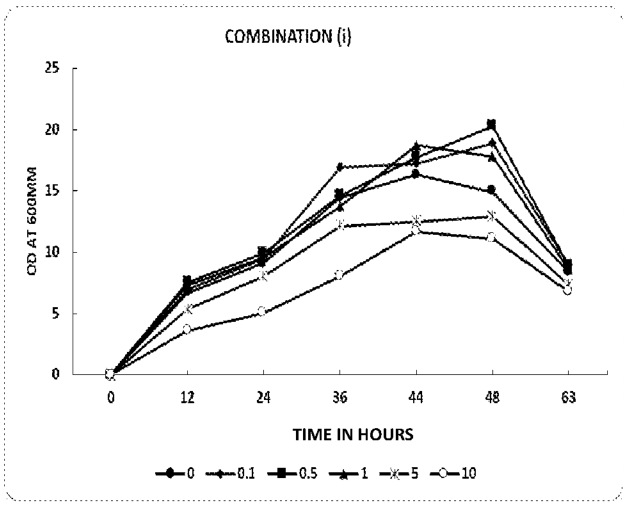

Fig. 11

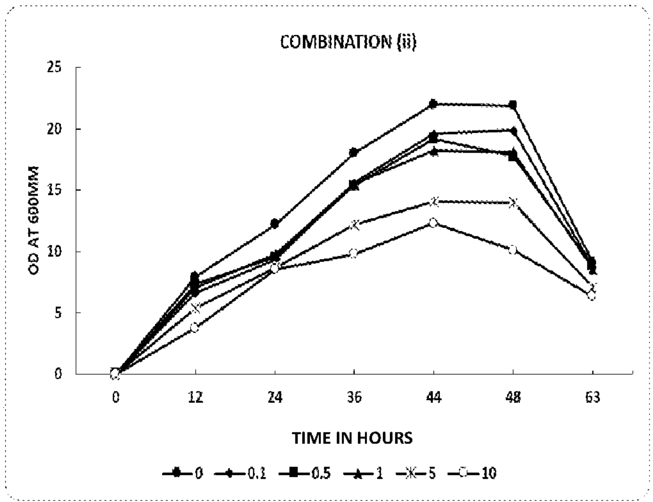

Fig. 12

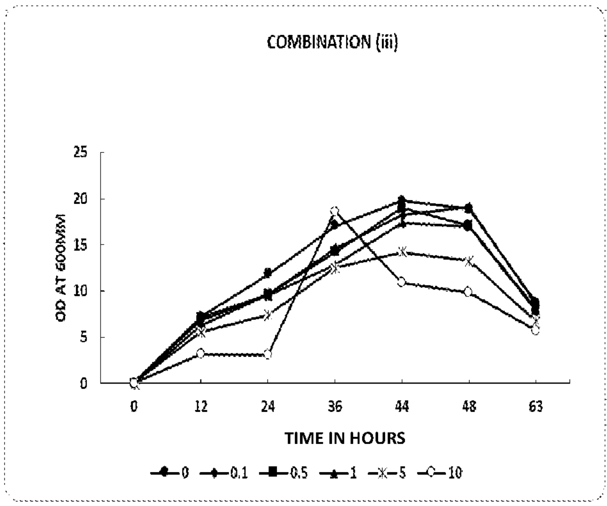

Fig. 13

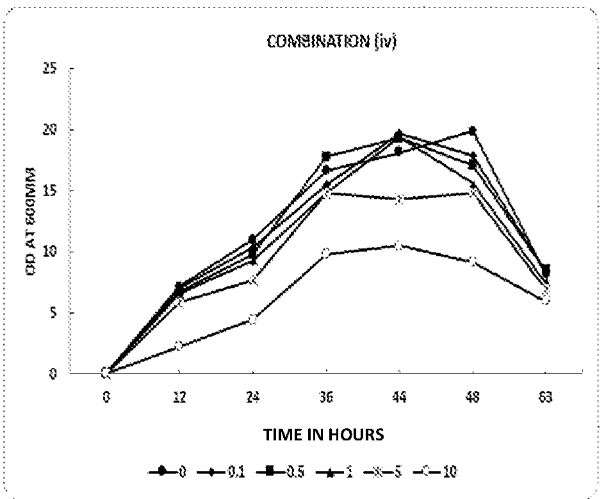

Fig. 14

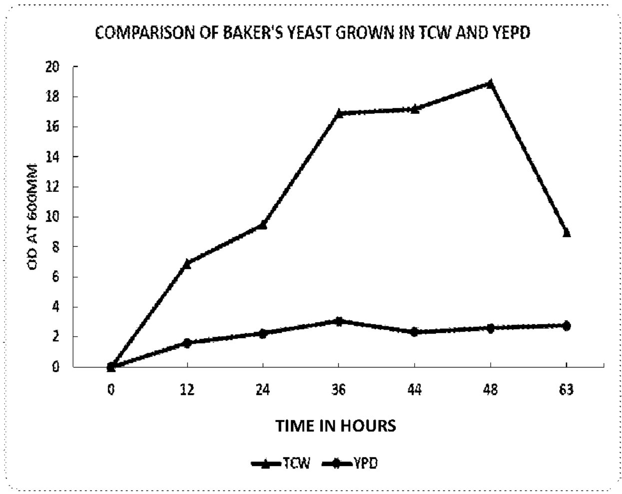

Fig. 15

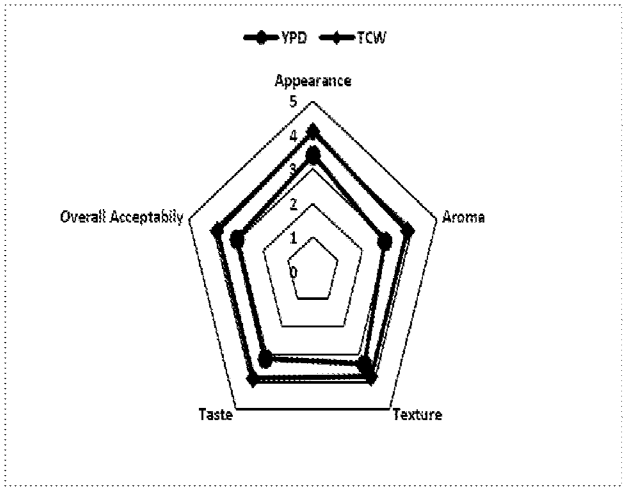

Fig. 16

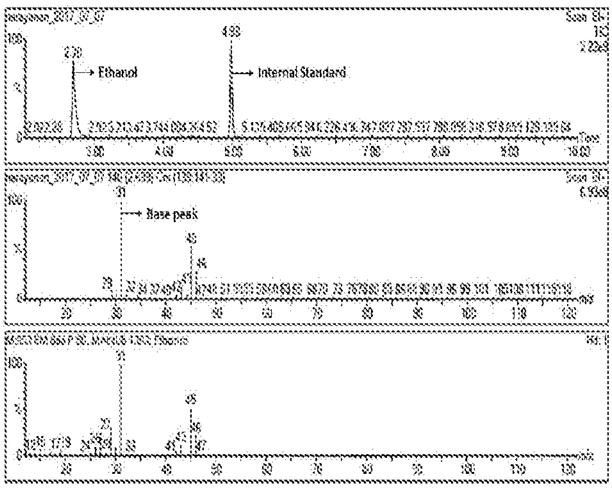

Fig. 17

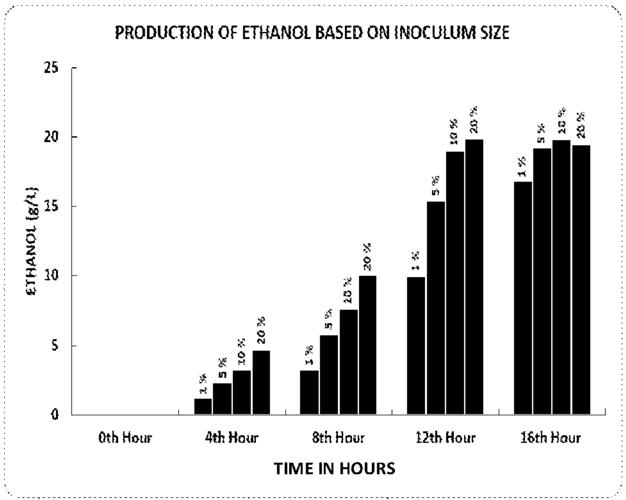

Fig. 18

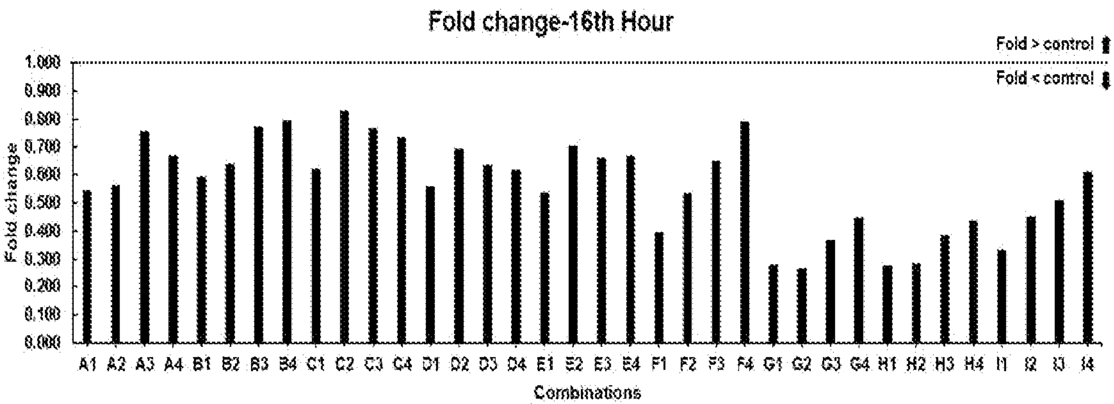

Fig. 19a

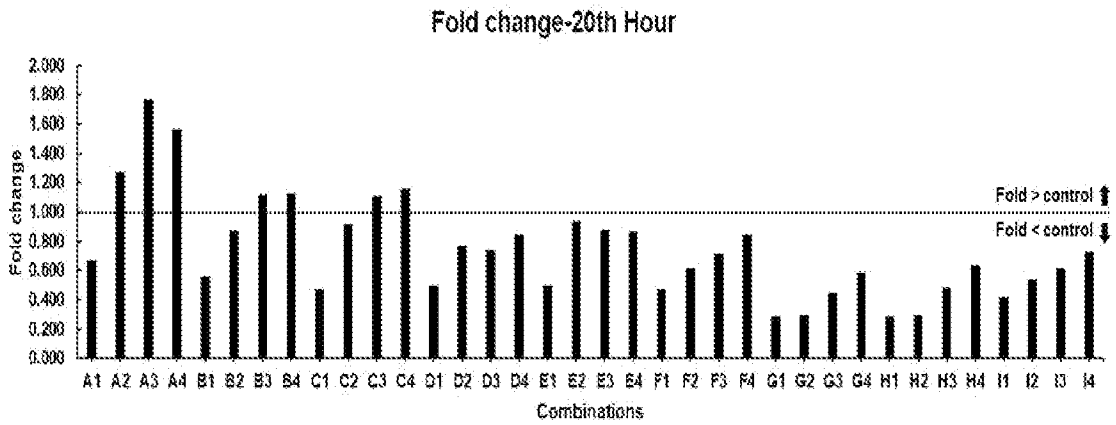

Fig. 19b

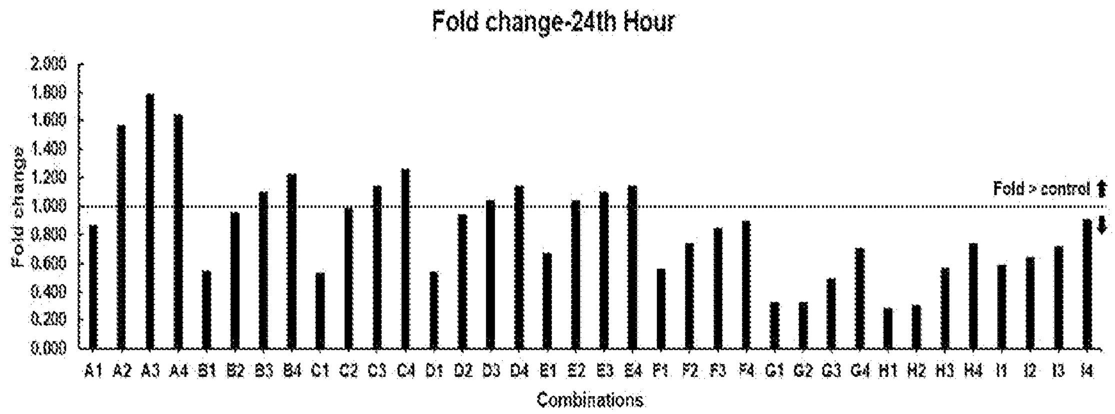

Fig. 19c

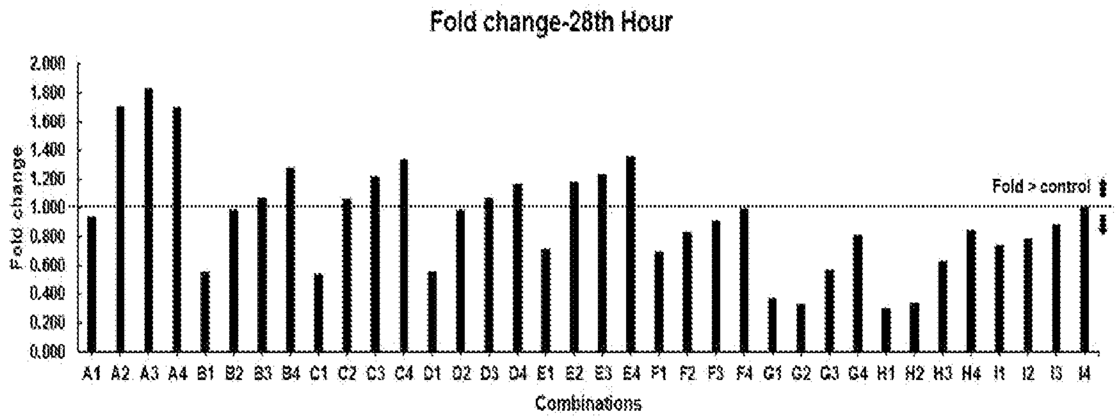

Fig. 19d

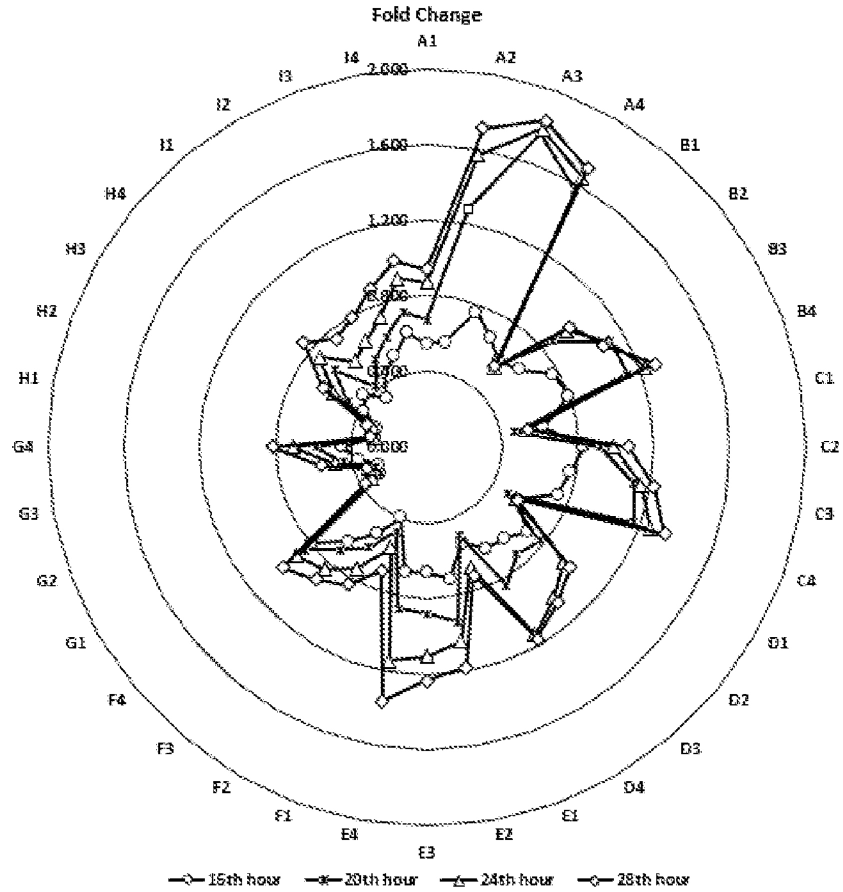
Fold Change
A1
A2
A3
A4
B1
B2
B3
B4
C1
C2
C3
C4
D1
D2
D3
D4
E1
E2
E3
E4
F1
F2
F3
F4
G1
G2
G3
G4
H1
H2
H3
H4
I1
I2
I3
I4
2.000
1.600
1.200
0.800
0.400
0.000
16th hour
20th hour
24th hour
28th hour

Fig. 19e

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IN2024/050326

A. CLASSIFICATION OF SUBJECT MATTER
C12N1/18,C12R1/865 Version=2024.01

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N, C12R

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database consulted during the international search (name of database and, where practicable, search terms used)

PatSeer, IPO Internal Database

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Narendrakumar Sekar et al. Tender coconut water an economical growth medium for the production of recombinant proteins in Escherichia coli, BMC Biotechnol. 2013; 13: 70. Whole document	1-14
Y	Whole document	15
Y	Neela Satheesh and NBL Prasad Production of fermented coconut water beverages, As. J. Food Ag-Ind. 2013, 6(05), 281-289 Page 283	15
Y	WO2014125429A1 (CENTRE FOR CELLULAR AND MOLECULAR PLATFORMS) 21 AUGUST 2014(21-08-2014) Whole document especially abstract and claims	15

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

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"D" document cited by the applicant in the international application

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25-07-2024	25-07-2024

Name and mailing address of the ISA/	Authorized officer
Indian Patent Office Plot No.32, Sector 14,Dwarka,New Delhi-110075 Facsimile No.	Anjana Haridas Telephone No. +91-1125300200

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/IN2024/050326

Citation	Pub.Date	Family	Pub.Date
WO 2014125429 A1	21-08-2014	US 20160002694 A1	07-01-2016
		MY 174102 A	10-03-2020
		SG 11201506342 XA	29-09-2015